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Our laboratory has shown that TNF can regulate normal mammary epithelial cell (MEC) growth, morphogenesis, and, under certain circumstances, functional differentiation in a manner similar to EGF. Since TNF has been shown to up-regulate EGF receptor (EGFR) expression and function in other systems, the current studies were undertaken to determine whether TNF action in MEC was indirect through stimulation of the EGFR. An inhibitor of EGFR tyrosine kinase activity, PD158780, failed to block proliferation induced by 40 ng/ml TNF and only partially inhibited growth in response to 2 ng/ml TNF. PD158780 was also unable to suppress the extensive morphological development induced by either TNF concentration. In contrast, the effects of TNF and PD158780 on functional differentiation (i.e. casein accumulation) were time-dependent. When measured on day 7 after 48 hours of treatment, casein accumulation was unaffected by either concentration of TNF or by PD158780. When assessed on day 21 after 16 days of treatment, however, casein levels were decreased by 40 ng/ml TNF and increased by PD158780. Significantly, this PD158780-induced increase in casein was not observed in MEC which had been treated with both PD158780 and TNF. These results thus suggest that EGFR tyrosine kinase activity is not necessary for TNF action in normal MEC.

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Linda M. Varela 7/18/97

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INTRODUCTION

The risk that a woman in the US will develop breast cancer in her lifetime has now increased to an unprecedented one out of eight women, and breast cancer is currently the most common cancer among women. It is therefore crucial that a better understanding of those factors leading to the development of breast cancer and of the metastatic phenotype be achieved so that more appropriate strategies for its prevention and/or treatment can be applied. This can be achieved by a thorough investigation of the normal mammary gland, and through a subsequent comparison of this with malignant breast cells, important clues as to how breast cancer evolves may be discovered. While the fundamental causes for breast cancer remain elusive, a growing body of data suggests that the major risk factors may be inherently biological, such as natural hormone or growth factor production (1). Thus, by examining the cyclical variations in hormone levels, the complex hormonal regulation of proliferation and differentiation, and the changes in gene expression that occur as the mammary gland progresses through the different stages of development, the prospects for prediction, prevention, and treatment of breast cancer may be heightened. A possible key player in this intricate network is tumor necrosis factor - alpha (TNFα). TNFα has been shown by our laboratory to play a significant role in directing both the proliferation as well as the morphological and functional differentiation of mammary epithelial cells (2). This regulator may reach the MEC via paracrine sources, and we have also shown that there is autocrine synthesis of TNFα by the MEC (3). Normally, there is strict control of the expression of this cytokine; however, it is possible that any disruption of this control has the potential to markedly affect the degree of both growth and differentiation and may confer on the cell a transformed phenotype. Thus, it is critically important to determine the physiological role of TNFα in the growth and development of the mammary gland and how this role differs in transformed cells, so that more appropriate strategies for the prediction, prevention, and treatment of breast cancer may be developed.

TNFα is a multifunctional cytokine that was originally defined by its ability to cause the hemorrhagic necrosis of tumors *in vivo*. It is now known, however, that TNFα affects the growth, differentiation, and/or function of virtually all cell types, either by acting alone or in concert with a variety of other cytokines, hormones or growth factors (4-7). On the first level, this complex physiology may be the result of different forms of TNFα, each of which has significant activity (8,9). Specifically, TNFα is first synthesized as a 26-kDa transmembrane precursor which is then proteolytically cleaved to release the 17-kDa soluble cytokine. In addition, the pleiotropic effects of TNFα may be mediated through its two distinct cell surface receptors of molecular masses 55- and 75-kDa (p55 and p75, respectively), which are expressed specifically and independently of each other in varying proportions on virtually all cells (10-12). Although the receptors have some sequence homology in their extracellular domains, they have no significant homology in their intracellular regions and have unique postreceptor signaling pathways which primarily induce distinct responses. For example, a large majority of TNFα activities, including cytotoxicity (13,14), antiviral activity (15), and stimulation of fibroblast proliferation (16), are mediated by p55, while the 75-kDa TNF receptor transduces signals for thymocyte proliferation (13), inhibition of hematopoiesis (17) and granulocyte macrophage-colony stimulating factor (GM-CSF) secretion (18). In some systems, however, the two receptors are also be capable of inducing the same effects. Agonistic antibodies directed against either TNF receptor were able to induce apoptosis in human rhabdomyosarcoma cells (19), and both TNF receptors share the ability to activate the

transcription factor NFκB; however, the time course of NFκB activation is different for each receptor, so it is believed that the signaling pathways of p55 and p75 may be distinct (20).

In contrast to the well-documented cytotoxic or cytostatic effects of TNFα in breast cancer cells (6,21,22), our laboratory has demonstrated that TNFα actually stimulates the growth and morphological development and regulates the function of normal mammary epithelial cells (MEC) in primary culture (2). These studies used a primary culture system in which undifferentiated rat MEC, cultured in the presence of a defined, serum-free medium, proliferate, morphologically develop and functionally differentiate to an extent comparable to that of cells within the mammary gland of a rat during lactation (23-25). Specifically, TNFα was found to stimulate MEC proliferation in both the presence and absence of epidermal growth factor (EGF), a major growth and differentiation factor for the MEC in this culture system. Both TNFα and EGF also stimulated the morphological development of the MEC organoids, inducing the formation of large, well-differentiated alveolar colonies which, in the case of TNFα, had extensive ductal branching (2). Lastly, TNFα modulated the functional differentiation (casein accumulation) of the MEC organoids in culture; however, these effects were more complex than its effects on either growth or morphogenesis. Higher concentrations of TNFα inhibited casein accumulation, but in the absence of EGF, lower concentrations of TNFα were found to stimulate casein accumulation at later times in culture, albeit to a lesser extent than EGF (2).

Studies performed in grant years 1 and 2 (July 1, 1994 through June 30, 1996)

Since these initial experiments suggested that TNFα may play a physiological role in normal mammary gland development, we believed that either the MEC or the stromal cells of the mammary gland would produce TNFα, one or both TNF receptors would be present on MEC, and that expression of both TNFα and its receptors would be developmentally (and thus hormonally) regulated. To answer these questions, I measured TNFα and TNF receptor expression in MEC isolated from mammary glands of rats during puberty, pregnancy, lactation, and post-lactational (day 7) involution. TNFα mRNA increased significantly during pregnancy, then decreased during lactation and involution. The 26-kDa, membrane-bound form of TNFα protein, undetectable in MEC from pubescent, virgin rats, increased throughout pregnancy and lactation and disappeared during involution. In contrast, p55 TNF receptor (TNFR) mRNA levels peaked in early lactation and declined thereafter, while p75 TNFR mRNA levels rose steadily through lactation. Additionally, I used specific agonistic antibodies directed against either the p55 or p75 TNF receptors to investigate the particular functions of each receptor in MEC in primary culture. The p55 TNFR was found to be the sole mediator of TNFα-induced proliferation, while the two receptors had opposing effects on functional differentiation (casein accumulation), with inhibition occurring through the p55 receptor and stimulation through p75. Taken together, these results suggested that TNFα plays a role in directing the growth and development of the mammary gland, and that both TNF receptors are important for TNFα function and may mediate different effects. These studies were published in *Endocrinology* 137: 4915-4924, 1996.

Studies performed in grant year 3 (July 1, 1996 through June 30, 1997)

Despite the knowledge gained by the studies completed in years 1 and 2, the postreceptor pathway(s) of TNFα action in normal MEC is still unknown. Indeed, it is not yet known whether

the actions of TNFα are direct or indirect via the induction of another cytokine or growth factor. One potential indirect signaling pathway may involve the EGF receptor (EGFR). Other groups have demonstrated that EGF receptors are present on normal MEC (26-28), and that MEC can synthesize various EGFR ligands, including TGFα (29,30), amphiregulin (31), and EGF (26,32). In addition, numerous studies have shown that TNFα can affect the EGFR on various levels, including mRNA expression, number and binding affinity of receptors, phosphorylation status, and tyrosine kinase activity (31,33-36), and TNFα can also induce the expression of various ligands of the EGFR, including TGFα and amphiregulin (31,34,37). Furthermore, Kalthoff et al. (34) have demonstrated that the two TNF receptors actually have diverse effects on EGF-ligand and -receptor family members, with signaling via p55 mediating the up-regulation of EGFR mRNA in epithelial cells and p75 transducing the signal for the up-regulation of TGFα mRNA.

Based on this information, I therefore hypothesized that signals from the TNF receptors might be up-regulating EGFR expression, ligand production and/or tyrosine kinase activity in MEC in order to indirectly stimulate MEC proliferation and/or differentiation. To test this latter hypothesis, I used a specific inhibitor of EGFR tyrosine kinase activity, PD158780 (compound 7f of Table 1 in reference (38)) in an attempt to block TNFα action on normal MEC in primary culture; these studies, which have just been accepted for publication in *Endocrinology*, are described in more detail below. PD158780 is a potent inhibitor of the tyrosine kinase activity of members of the EGFR family, which includes the EGFR itself as well as erbB2, B3 and B4, but has poor inhibitory activity against the PDGF or FGF receptor tyrosine kinases (Dr. David Fry, personal communication).

Address to Statement of Work:

The following aspect of the Statement of Work was addressed:

A. *Investigation of the functional pathways of TNF receptor action in normal MEC in primary culture.* The purpose of these studies was to further investigate the functional roles of the TNF receptors in normal MEC by examining potential pathways of TNF receptor action. Specifically, the role of the EGF receptor as an indirect mediator of TNFα action was studied.

EXPERIMENTAL METHODS AND PROCEDURES

Materials

Phenol red-free RPMI-1640, newborn calf serum (NCS), and gentamicin were purchased from Life Technologies (Grand Island, NY), collagenase class III was purchased from Worthington Biochemical (Freehold, NJ), dispase grade II powder (neutral protease) was a product of Boehringer-Mannheim (Indianapolis, IN), and liquid dispase (50 caseinolytic units/ml) was purchased from Collaborative Biomedical Products (Bedford, MA). Phenol red-free Dulbecco's Modified Eagle's Medium-F12 (DMEM-F12) (1:1, containing 15 mM HEPES), NP-40, insulin, progesterone, hydrocortisone, ascorbic acid, apo-transferrin, fatty acid-free bovine serum albumin (BSA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) were from Sigma Chemical Co. (St. Louis, MO). Donkey anti-rabbit peroxidase-conjugated immunoglobulin G was a product of Jackson ImmunoResearch Labs. (West Grove, PA). Mouse epidermal growth factor (EGF) was purchased from Upstate Biotechnology (Lake Placid, NY), and ovine prolactin (NIDDK-oPL-20) was a gift of NIDDK-NIH (Bethesda, MD). [Methyl-^{3}H]-thymidine was purchased from DuPont NEN (Boston, MA). Recombinant human TNFα (2.5 x 10^6 U/mg) was a generous gift of Asahi Chemical Industry Co. (Fuji, Shizuoka, Japan), and the inhibitor of EGFR tyrosine kinase activity, PD158780, was kindly provided by Dr. David Fry at Parke-Davis (Ann Arbor, MI).

Animals

For the isolation of mammary epithelial cells (MEC) for primary culture, virgin, 50- to 55-day-old female Sprague-Dawley Crl:CD BR rats were purchased from Charles River (Wilmington, MA) and used as the source of mammary glands. Female CD2F1 mice, purchased from NCI-Frederick Cancer Research Facility, Biological Testing Branch (Frederick, MD), were used to passage the Engelbreth-Holm-Swarm (EHS) sarcoma, which was the source of the reconstituted basement membrane (RBM) matrix for the primary culture of the MEC organoids. Rats and mice were fed chow diets (Teklad, Madison, WI) *ad libitum* and had free access to water. Animal rooms were humidity controlled and air conditioned, with light cycles of 14 hrs on-10 hrs off (rats) or 12 hrs on-12 hrs off (mice). The care and use of the animals was in accordance with NIH guidelines and Institute Animal Care and Use Committee regulations.

RBM matrix preparation

The RBM matrix used for the primary culture of the MEC organoids was extracted from the EHS sarcoma as previously described (23).

Mammary epithelial cell isolation and cell culture

For the primary culture of MEC, epithelial cell organoids were isolated and cultured as previously described. Briefly, mammary glands were excised from virgin, 50- to 55-day old female rats (8 per experiment), mechanically minced, suspended in 10 ml/g wet wt digestion solution (phenol red-free RPMI 1640 containing 0.2% (w/v) dispase II, 0.2% (w/v) collagenase type III, 5% (v/v) NCS, and 50 μg/ml gentamicin) and incubated at 37°C for approximately 13.5 hours. The resultant epithelial cell organoids were pelleted by centrifugation, washed twice with DMEM-F12, and resuspended in DMEM-F12. The MEC suspension was then filtered through a 530-μm nitex filter (Tetko, Depew, NY) to remove any large aggregates of glandular

material and subsequently passed through a 60-μm nitex filter, which trapped the MEC organoids but allowed the passage (and removal) of small cell clusters and single cells. The organoids were rinsed off the nitex filter with DMEM-F12 (phenol red-free) containing 5% (v/v) NCS and 50 μg/ml gentamicin, refiltered through the 530-μm nitex, and incubated in a plastic tissue culture flask for 4 hrs at 37°C; this step facilitated the attachment and removal of any remaining stromal cells from the non-adherent mammary epithelial organoids. The number of MEC within the organoids was then enumerated by counting of nuclei as previously described (23). The non-adherent MEC organoids were pelleted by centrifugation and resuspended in ice-cold RBM matrix at a concentration of 3×10^5 cells per 0.2 ml matrix. Two hundred microliters of RBM matrix containing epithelial organoids was layered on top of 200 μl pre-gelled, cell-free RBM matrix per well of a 24-well plate. After the matrix was allowed to gel for 3 hours at 37°C, one milliliter of medium was added to each well. The medium was changed on day 5 as indicated below; thereafter, the medium was changed twice per week. The serum-free medium used for these studies consisted of 10 μg/ml insulin, 1 μg/ml progesterone, 1 μg/ml hydrocortisone, 5 μg/ml transferrin, 1 μg/ml prolactin, 5 μM ascorbic acid, 1 mg/ml fatty acid-free BSA, and 50 μg/ml gentamicin in phenol red-free DMEM-F12. EGF (10 ng/ml; 1.6 nM) or TNFα (2 or 40 ng/ml; 0.1 or 2.3 nM, respectively) were added where noted in the text. PD158780 was dissolved in 0.1% DMSO and added to the media immediately prior to use.

^{3}H-Thymidine incorporation assay

The MEC organoids were cultured until day 5 in the EGF- and TNFα-free medium described above. On day 5, the medium was changed and EGF (10 ng/ml; 1.6 nM) or TNFα (2 or 40 ng/ml; 0.1 or 2.3 nM, respectively) in either the presence or absence of the EGFR tyrosine kinase inhibitor PD158780 (0.5 μM) were added, and the cultures were incubated for either 48 hrs (until day 7) or 16 days (until day 21) at 37°C. As a control for these studies, PD158780 alone was added to the EGF- and TNFα-free culture medium. After the cultures were pulse-labeled with [^{3}H]-thymidine (5 μCi/well) for the last 4 hours of incubation on either day 7 or day 21, the medium was removed, and the RBM matrix was digested away using 5 caseinolytic units/ml liquid dispase for 2 hours at 37°C. The MEC organoids were washed with cold PBS, and the acid-insoluble fraction was precipitated overnight at 4°C with 1 ml of 5% (w/v) TCA. The pellets were washed twice with cold 5% TCA, solubilized in 1 ml of 0.1N NaOH containing 0.1% (v/v) Triton X-100, neutralized with 100 μl of 1N HCl, and [^{3}H]-thymidine incorporation was determined by liquid scintillation counting.

Quantitation of cell number (MTT assay)

Culture conditions and treatment were as described above for the [^{3}H]-thymidine incorporation assay. Cell number was quantitated on days 7 and 21 using the MTT assay that we have previously described (2). In this assay, viable cells convert the soluble tetrazolium MTT dye into insoluble blue formazan crystals, while non-viable cells are unable to metabolize the MTT substrate, and thus are not counted. The total, viable cell number is then determined by extrapolation of the absorbance intensity from a standard curve that is constructed for each experiment. In brief, 200 μl of MTT (5 mg/ml in PBS) was added per ml of medium, and cultures were incubated for 16 hrs at 37°C. After removal of the medium and rinsing of each well with PBS, the RBM matrix was digested by adding 1 ml of dispase (5 caseinolytic units/ml) per well and incubating for 2 hrs at 37°C. The digested material was transferred to glass tubes, and each well was rinsed with 1 ml of PBS. The cells were then separated from the

digested matrix by centrifugation, and the pellet was dissolved in isopropanol and recentrifuged. Absorbance at 570 nm was read in a Bio-Tek model EL311 plate reader (Winooski, VT). Standard curves using the newly isolated MEC were set up for each experiment. It was also established that PD158780 did not interfere with the MTT assay, as nuclei counts at day 21 did not differ significantly from cell counts obtained using the MTT assay (data not shown).

Morphological analysis

Culture conditions and treatment were as described above for the [^{3}H]-thymidine incorporation assay. The morphological appearance of the MEC organoids was assessed and quantitated on days 7 and 21 of culture (during the last 4-6 hours of treatment) by light microscopic observation. Colonies were classified into four main groups: end bud-like, alveolar, squamous and atypical hybrid (25). The end bud-like colonies are pale-rust in color, have a more simplistic lobular structure with fewer, smaller ductal projections, and are primarily composed of immature epithelial cells which show little or no evidence of functional differentiation. In contrast, the dark brown or black alveolar colonies are larger, have a more complex, multilobular structure with extensive ductal projections and are composed of morphologically and functionally differentiated MEC organized into a classical alveolar arrangement. Squamous colonies contain concentrically compacted acellular material in a keratotic whorl pattern (2,39), and atypical hybrid colonies are defined as hybrids of alveolar and squamous colonies. Photographs of the organoids were taken with a Nikon FX-35A camera mounted on an Olympus CK2 inverted microscope (Melville, NY).

Assessment of functional differentiation (measurement of casein accumulation)

Culture conditions and treatment were as described above, and samples were harvested on days 7 and 21 of culture as previously described (2). Casein levels were assayed in three wells per treatment group using the standard casein ELISA previously developed by our laboratory (24). In addition, casein accumulation by MEC was determined using a modification of a recently described Western blot procedure (3). In this case, samples were mixtures of the triplicate wells for each treatment group. The samples were normalized for loading on the basis of either equivalent protein contents (following Bio-Rad protein analysis) or equivalent cell number. In either case, extracts of MEC plus RBM matrix were diluted with 4-fold concentrated Laemmli sample buffer (40), boiled for 4 minutes, subjected to electrophoresis on a 12.5% polyacrylamide-SDS gel according to the method of Laemmli (40), and transferred to nitrocellulose. After the membranes were blocked overnight at 4°C with 5% (w/v) Blotto (nonfat dried milk) in TBS buffer (150 mM NaCl, 10 mM Tris, pH 7.4 at 25°C), they were rinsed in TBS containing 0.5% (w/v) BSA (TBS/BSA) and incubated for 2 hrs at room temperature with a rabbit polyclonal antibody against the rat casein proteins (24) (1:3000 dilution) in TBS/BSA. Blots were then washed in TBS/BSA, incubated with peroxidase-conjugated donkey anti-rabbit antiserum (1:5000 dilution in TBS/BSA) for 60 min at room temperature, washed in TBS/BSA containing 0.01% (v/v) Tween 20, and developed using the enhanced chemiluminescence system (Amersham).

Statistics

Statistical significance was determined using a one-way analysis of variance (ANOVA) with the Student-Newman-Keuls test for pairwise multiple comparisons. $P < 0.05$ was judged to be statistically significant.

RESULTS

Effect of PD158780 on the TNFα-induced proliferation of normal MEC

Preliminary studies which evaluated the effects of various concentrations of the EGFR tyrosine kinase inhibitor on MEC indicated that at 0.5 μM, PD158780 was inhibitory to EGF-induced effects but was not cytotoxic to the cells (K.M. Darcy, manuscript in preparation). Thus, this concentration of PD158780 was used in an attempt to inhibit the effects of TNFα on normal MEC growth and differentiation.

First, the ability of PD158780 to block TNFα-induced DNA synthesis in normal MEC was investigated using a ^{3}H-thymidine incorporation assay. When assessed on day 7 of culture after 48 hours of treatment, the EGFR tyrosine kinase inhibitor only modestly suppressed the increase in ^{3}H-thymidine incorporation stimulated by 40 ng/ml TNFα (Figure 1, bars 5 and 6) but reduced DNA synthesis in response to 2 ng/ml TNFα by approximately 50% (Figure 1, bars 7 and 8). In contrast, the EGF-induced increase in DNA synthesis was completely blocked by PD158780 (Figure 1, bars 3 and 4). Moreover, the EGFR tyrosine kinase inhibitor alone was found to cause a 50% inhibition of ^{3}H-thymidine incorporation. Since no EGF was present in the culture medium, we believe that this inhibition was due to the blockage of the activity of endogenous EGFR ligands either produced by the MEC themselves or present in the RBM matrix.

The MEC culture system utilized herein also permits both morphological and functional differentiation over a 3 week period. In order to determine whether the pathway of TNFα action changed as the MEC differentiated, the cells were also treated until the end of the 21 day culture period (for a total of 16 days). When measured on day 21, ^{3}H-thymidine incorporation by MEC treated with either concentration of TNFα was not increased relative to the control as it had been on day 7 (Figures 1 and 2, compare bars 5 and 7 to bar 1). However, it can be seen that PD158780 still had no effect on DNA synthesis in the presence of 40 ng/ml TNFα (Figure 2, compare bars 6 and 5), but again caused a 50% inhibition at the lower, 2 ng/ml concentration (Figure 2, compare bar 8 with bar 7). Of considerable interest was the marked difference in thymidine incorporation between TNFα- and EGF-treated MEC at this time. In contrast to both TNFα-treated groups and to day 7, thymidine incorporation by MEC grown in the presence of EGF for 16 days was dramatically decreased compared to the control group; moreover, PD158780 had no further inhibitory effect on DNA synthesis in the presence of EGF. Also noteworthy was the marked inhibitory effect of PD158780 on thymidine incorporation in the absence of added growth factor (Figure 2, compare bars 2 and 1).

Concurrently, the total, viable cell number was also quantitated in the various treatment groups in order to determine the balance between cell growth and death in response to TNFα, and to determine whether this balance was affected by inhibition of EGFR tyrosine kinase activity. In contrast to the stimulatory effect on thymidine incorporation, neither concentration of TNFα, in either the presence or absence of PD158780, had any effect on viable cell number after 48 hours (day 7). Cell number was increased in the EGF-treated group, however, and this increase was completely suppressed by PD158780, while cell number was unaffected by the 48 hour treatment with PD158780 alone (Figure 3).

When assessed on day 21 after 16 days of treatment, the viable cell number in both TNFα-treated groups was increased when compared to day 7 and was significantly higher than the

cell number in the EGF-treated culture (Figure 4, compare bars 5 and 7 to 3). More significantly, PD158780 was unable to block the increase in viable cell number induced by 40 ng/ml TNFα (Figure 4, compare bars 5 and 6), but partially suppressed the increase in cell number in response to 2 ng/ml TNFα. Finally, the EGF-induced increase in viable cell number was blocked by PD158780 (Figure 4) as it had been at day 7, while the EGFR tyrosine kinase inhibitor alone had no effect (compare bars 1 and 2). Thus, when taken together, these data suggest that EGFR tyrosine kinase activity is not required for TNFα to induce the proliferation of normal MEC in culture, although the pathway(s) activated by both the TNFα and EGF receptors may act in concert to stimulate MEC growth under certain circumstances.

Effect of PD158780 on TNFα-induced morphological differentiation

We have previously shown that both TNFα and EGF can stimulate the ductal and alveolar morphogenesis of MEC in culture while suppressing the outgrowth of colonies with an atypical or squamous epithelial morphology (2,39). In order to determine whether the EGFR was involved in the pathway by which TNFα stimulated the morphological development of the MEC, the ability of PD158780 to inhibit TNFα-induced morphogenesis was assessed. Morphological changes after 48 hours of treatment were subtle (data not shown). In both TNFα- and EGF-treated groups, the percentage of the less-differentiated, end bud-like colonies was slightly decreased while the percentage of the more-differentiated, alveolar colonies was increased; the percentage of squamous and atypical hybrid colonies in all groups was negligible at this time. The EGFR tyrosine kinase inhibitor had no significant effect on colony morphology in either the presence or absence of TNFα or EGF.

In contrast, TNFα induced significant changes in both colony type and size after 16 days of treatment. Specifically, 40 ng/ml TNFα stimulated the formation of complex, lobulo-alveolar colonies which were significantly larger than those induced by EGF (Figure 5, compare j with f and g) and which were interconnected by extensive ductal branching (Figures 5j and 6). Furthermore, TNFα also inhibited the development of both squamous and atypical colonies (Figure 6). More importantly, the EGFR tyrosine kinase inhibitor was unable to inhibit this expansive TNFα-induced morphogenesis, as colonies that developed in the presence of TNFα plus PD158780 were as large, viable and morphologically developed as those which developed in the presence of TNFα alone (Figures 5j, 5k and 6). The 2 ng/ml concentration of TNFα also stimulated alveolar and ductal morphogenesis, although the effects were less pronounced than those of 40 ng/ml TNFα, and PD158780 was still unable to block this TNFα-induced morphological differentiation ((2) and data not shown).

In contrast to its inability to alter morphological development in the presence of TNFα, the EGFR tyrosine kinase inhibitor had several noteworthy effects on MEC morphogenesis when added alone or in combination with EGF. Unexpectedly, PD158780 alone mimicked the ability of EGF to both suppress squamous and atypical colony formation and to stimulate the formation of the more differentiated, lobular-alveolar colonies (Figure 6). These latter colonies, however, tended to be quite small in comparison to TNFα- or EGF-treated colonies (Figure 5d), and any larger alveolar colonies which developed in the presence of PD158780 alone appeared to be highly disrupted and were surrounded by small cellular bodies which had apparently broken off of the periphery of the alveolar organoids (Figure 5e). Furthermore, organoids treated with both EGF and PD158780 were not as large as colonies treated with

EGF alone (Figure 5, compare h and i to g), and many of the alveolar colonies in this group also appeared to be highly disrupted (Figure 5i).

Effect of PD158780 on TNFα-modulated MEC functional differentiation

Previous studies by our laboratory determined that TNFα had a complex, biphasic effect on casein production by MEC: a higher concentration of TNFα (40 ng/ml) inhibited functional differentiation, while in the absence of EGF, a lower concentration of TNFα (2 ng/ml) enhanced casein accumulation (2). To determine whether these effects of TNFα might be mediated through the EGFR, the ability of PD158780 to interfere with the effects of TNFα on casein accumulation by MEC was measured using both Western blot analysis and an ELISA (24). When examined on day 7, casein accumulation was found to be unaffected by the 48 hour treatment with either 2 or 40 ng/ml TNFα (Figure 7, lane 5 and data not shown), and casein levels were also unchanged in MEC which had been treated with both TNFα and PD158780 (Figure 7, lane 6 and data not shown). The EGFR tyrosine kinase inhibitor completely blocked the increased accumulation of all casein isoforms in response to EGF (Figure 7, compare lanes 3 and 4), while PD158780 alone had no significant effect on casein levels (Figure 7, compare lanes 2 and 1). Casein levels in all but the EGF-treated group were below the limits of detectability by ELISA (data not shown).

When measured on day 21, however, casein levels were found to be significantly decreased by the 16 day treatment with 40 ng/ml TNFα, and the EGFR tyrosine kinase inhibitor had no effect on this TNFα-induced inhibition (Figure 8A, compare lanes 1, 5 and 6, and Figure 8B). Despite its ability to significantly stimulate both MEC growth and morphogenesis, 2 ng/ml TNFα had no effect on casein levels after 16 days of treatment even though this TNFα concentration had previously been shown to increase casein accumulation by MEC at later times in culture (Figure 8A, lanes 1 and 7, and Figure 8B). Casein levels were also unchanged in MEC which had been treated with both 2 ng/ml TNFα and PD158780.

Unexpectedly, casein accumulation was *increased* in MEC which had been treated for 16 days with PD158780 alone or with both EGF and PD158780 (Figure 8A and 8B). This latter effect was in direct contrast to the inhibitory effect of PD158780 on EGF-induced casein accumulation after 48 hours. Lastly, it should also be noted that this PD158780-induced increase in casein accumulation was not observed in the presence of either concentration of TNFα (Figure 8A and 8B).

DISCUSSION

EGFR tyrosine kinase activity is not necessary for TNFα-induced MEC proliferation

The results of our previous studies indicated that the combined mitogenic action of TNFα and EGF on normal MEC was less than additive (2). This suggested that TNFα and EGF might be activating a common growth stimulatory pathway, and that the mitogenic effect of TNFα might be mediated, at least in part, via activation of the EGFR in response to signals from the p55 TNF receptor. On the other hand, this earlier data also suggested that the mitogenic signaling pathway triggered by the p55 TNF receptor may have an EGFR-independent component, since TNFα was shown to increase total cell number to a higher level than that observed with optimal levels of EGF [(2,23,39); Figure 4]. The studies presented herein suggest that EGFR tyrosine kinase activity is not required for TNFα to stimulate MEC proliferation. PD158780 was unable to inhibit proliferation in response to 40 ng/ml TNFα and only partially suppressed the growth promoting effects of 2 ng/ml TNFα. Although we cannot rule out the possibility that PD158780 may be inhibiting an, as yet undiscovered, tyrosine kinase which is activated by 2 ng/ml TNFα, this latter observation suggests that signaling pathway(s) activated by endogenous EGFR ligands may act in concert with pathways activated by the lower concentration of TNFα to stimulate growth. In support of this theory, EGF and TNFα have been shown to preferentially activate different members of the mitogen-activated protein (MAP) kinase family in other cell types. EGF strongly stimulates ERK1 and ERK2 activity, while TNFα induces a more pronounced activation of the c-Jun N-terminal kinases (JNKs) (41-43). Thus, when EGFR activation is blocked by PD158780 such that ERK1 and/or ERK2 may no longer be activated by this receptor, growth in the presence of 2 ng/ml TNFα may be partially, but not completely, suppressed.

In contrast, the higher (40 ng/ml) concentration of TNFα may be able to directly activate ERK1 and/or ERK2 such that the blockage of endogenous EGFR ligand-induced action by PD158780 has no effect on DNA synthesis in response to this TNFα concentration. Even though TNFα is not as powerful a stimulus for ERK1 and ERK2 as EGF, numerous studies have demonstrated that TNFα can activate these enzymes (44-47), so it could be proposed that the higher concentration of TNFα may activate both the JNK and ERK enzymatic signaling cascades to stimulate MEC growth. Furthermore, a study by Sluss et al. (48) has demonstrated that there are two JNK protein kinase isoforms, JNK1 and JNK2, and that the activation of these protein kinases by TNFα is concentration-dependent. Therefore, it could be postulated that 40 ng/ml TNFα activates both JNK1 and JNK2, while the lower TNFα concentration only triggers the activation of one JNK isoform. The higher TNFα concentration may also preferentially activate JNK2, which has been shown to be more potent in inducing the phosphorylation of c-jun than JNK1 (48). To begin to answer these questions, I am currently investigating the specific roles of both of the ERK and JNK MAP kinase isoforms in modulating MEC growth in response to TNFα.

The differential effects of TNFα and EGF on MEC proliferation

The distinct effects of TNFα and EGF on MEC proliferation after different times in culture were intriguing. When assessed on day 7, thymidine incorporation, but not cell number, was increased in response to TNFα, while both DNA synthesis and cell number were increased by EGF. This observation further supports our contention that the pathways of TNFα- and EGF-

induced growth in normal MEC are different. In addition, this difference in cell number between the TNFα- and EGF-treated groups may be due to the ability of TNFα to increase the overall rate of cell turnover at this time. Since several studies have demonstrated that TNFα can be either cytotoxic or mitogenic to certain cell types (49,50), it is possible that in addition to an overall stimulation of MEC proliferation, TNFα may be cytotoxic to a specific subset of MEC within the cultures, such that there is no net increase in cell number. However, once the MEC to which TNFα is cytotoxic have been eliminated, the proliferative effects of TNFα may become apparent. EGF, in contrast, may have no effect on the rate of cell turnover at this time; thus, its growth stimulatory effects are immediately evident.

In contrast to day 7, the total cell number on day 21 in the groups treated with either 2 or 40 ng/ml TNFα was significantly higher than that in the EGF-treated cultures (Figure 4). Of further interest, however, was the observation that MEC cultured in the presence of either concentration of TNFα were still actively synthesizing DNA at this time (Figure 2), albeit at a reduced level when compared to day 7, whereas DNA synthesis by EGF-treated MEC was significantly lower than the level in TNFα-treated cells. The level of ^{3}H-thymidine incorporation by the EGF-treated cells on day 21 was also significantly lower than the level on day 7 and was actually 3.5 fold lower than thymidine incorporation by MEC cultured in the absence of EGF or TNFα. This latter observation suggests that EGF may be acting to inhibit cell growth at this time; however, the fact that PD158780 did not reverse this inhibition argues against this supposition. Rather, the data suggest a loss of responsiveness of the EGF-treated cells to EGF, perhaps by down-regulation of EGFR activity in response to the 16 day EGF treatment. Alternatively, or perhaps in addition, several groups have reported that EGFR levels decrease during pregnancy and lactation [(27,28) and K.M. Darcy, manuscript in preparation], and we have recently determined that EGFR levels decline in conjunction with the morphological and functional differentiation of MEC in primary culture (K.M. Darcy, manuscript in preparation).

In any case, the different effects of TNFα and EGF on DNA synthesis and cell number at different times in culture strongly suggest that the mitogenic actions of TNFα and EGF are mediated through independent pathways, even though there may be cooperativity between these pathways under some circumstances. TNFα may also be acting on a cell population that is unresponsive to EGF. For example, both TNFα and EGF may be able to regulate the growth and differentiation of immature MEC, while TNFα may also be able to stimulate the proliferation of a putative stem cell population and/or effect the death of a small subset of cells early in culture.

The EGFR tyrosine kinase inhibitor did not block the TNFα-induced morphological development of normal MEC

The extensive branching alveolar morphogenesis that was stimulated by both concentrations of TNFα was completely unaffected by PD158780, suggesting that the pathway of TNFα-induced morphological development is not dependent on EGFR tyrosine kinase activity. This conclusion is further supported by the observation that TNFα actually induced the formation of larger colonies with more expansive ductal branching than EGF (Figure 5). This difference between TNFα- and EGF-stimulated morphogenesis may be due to the differential ability of TNFα and EGF to modulate the production and/or activity of matrix metalloproteinases (MMPs) which could then affect the remodeling of the ECM. Previous studies in our laboratory have shown that MMP-9 (the 92 kDa type IV collagenase) activity in

conditioned medium from MEC was increased by TNFα but decreased by EGF [(51) and unpublished observations]. Therefore, the TNFα-induced remodeling of the ECM may permit the pronounced ductal branching and alveolar morphogenesis that occurs in response to this cytokine, while the inability of EGF to increase MMP-9 activity may explain why EGF does not stimulate branching morphogenesis to the same extent as TNFα.

In addition, it should also be noted that PD158780 alone had several interesting morphological effects. Earlier studies in our laboratory have shown that in the absence of EGF, branching alveolar morphogenesis was decreased and both atypical hybrid and squamous colony formation was enhanced (2,39). In the current studies, however, treatment of the MEC with an inhibitor of EGFR tyrosine kinase activity actually permitted alveolar morphogenesis, although the PD158780-treated colonies were smaller than those treated with either EGF or TNFα. Since PD158780 was not present for the first 5 days of culture, endogenous EGFR ligands produced by the MEC or within the ECM may have initiated the process of morphological differentiation such that the subsequent inhibition of EGFR tyrosine kinase activity was unable to inhibit morphogenesis once it had begun. This hypothesis concurs with previous studies in our laboratory in which alveolar morphogenesis proceeded even when EGF was removed from the culture medium after the first few days of culture (39). However, PD158780 also induced the apparent disintegration of the alveolar colonies when added alone or in combination with EGF (but not in the presence of TNFα), so even though PD158780 did not inhibit alveolar morphogenesis, inhibition of the EGFR tyrosine kinase activity resulted in the apoptotic death of the lobulo-alveolar organoids (52). When taken together with the aforementioned data on the effects of EGF on DNA synthesis at different times, as well as with earlier studies by both our laboratory (39) and Nancy Hynes group (53), it thus appears that EGF is needed at early times in culture (e.g. day 7) for proliferation as well as for rendering MEC competent to respond to lactogenic hormones. After differentiation, however, EGF may become a survival factor which serves to prevent apoptosis and maintain the existing state of the gland rather than stimulate further growth and/or development.

The EGFR tyrosine kinase inhibitor did not block the effects of TNFα on MEC functional differentiation

In accordance with our previous studies, casein accumulation by MEC was inhibited by a 16 day treatment with 40 ng/ml TNFα. Since PD158780 was unable to alter this effect, it appears that the EGFR is not involved in the pathway whereby 40 ng/ml TNFα regulates casein accumulation. In contrast to our previous studies, casein accumulation was not increased after long-term treatment with 2 ng/ml TNFα. This apparent discrepancy may be due to the fact that TNFα was not added until day 5 in the current study, while TNFα had been present from days 0-21 of culture in the former studies. In addition, the stimulatory effect of 2 ng/ml TNFα on casein may have been masked because casein levels in the control group were unusually high in this series of experiments; this phenomenon may be due to the activity of endogenous EGFR ligands either produced by the MEC themselves or present in the RBM matrix.

Surprisingly, casein accumulation was *increased* in MEC which had been treated for 16 days with PD158780 alone or in combination with EGF. Since studies by our laboratory have shown that casein accumulation by MEC is significantly decreased in the absence of EGF, this observation was somewhat unexpected. It could be argued that this PD158780-induced increase in casein may be due, in part, to the 2-fold increase in the percentage of the alveolar, casein-producing colonies in this group; however, several factors argue against this

explanation. First, in both EGF and EGF plus PD158780 treated cultures, the percentage of alveolar colonies was increased to the same extent compared to the control group, yet casein levels were higher in MEC treated with both EGF and PD158780 than in MEC treated with EGF alone. In addition, TNFα also increased the percentage of alveolar colonies, but casein accumulation by TNFα-treated MEC was either decreased or unaffected, depending on the TNFα concentration. Therefore, the increased percentage of alveolar colonies cannot completely account for the increase in casein levels in the PD158780-treated cultures.

In summary, the studies presented herein suggest that the modulation of MEC growth and development by TNFα does not require EGFR tyrosine kinase activity. Specifically, EGFR tyrosine kinase activity is not necessary for TNFα to stimulate the proliferation of normal MEC in culture; however, low concentrations of TNFα may act in concert with EGF to stimulate MEC growth. In addition, the EGFR tyrosine kinase inhibitor had no effect on the ability of TNFα to either stimulate MEC morphogenesis or regulate casein accumulation by MEC. Further studies are currently underway in order to determine the particular mechanisms whereby signals from the TNF receptors regulate MEC growth and differentiation.

CONCLUSIONS

1. The EGFR tyrosine kinase inhibitor did not block MEC proliferation induced by 40 ng/ml TNFα, but partially supressed growth induced by 2 ng/ml TNFα. This suggests that EGFR tyrosine kinase activity is not required for TNFα-induced MEC proliferation; however, the pathway(s) activated by the p55 TNF receptor and the EGFR may act in concert to stimulate MEC growth under some conditions.

2. The EGFR tyrosine kinase inhibitor did not block the TNFα-induced morphological development of normal MEC in primary culture.

3. The EGFR tyrosine kinase inhibitor had no effect on the ability of TNFα to regulate casein accumulation by MEC.

4. These studies also suggest that the role of EGF may change during normal MEC development.

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APPENDICES

I. FIGURES

see attached pages

II. FIGURE LEGENDS

Figure 1. The effects of EGF, TNFα and PD158780 on thymidine incorporation by MEC on day 7 of culture. MEC were cultured in serum-free medium lacking EGF, TNFα and drug (control) until day 5; the medium was then changed and the MEC were treated as indicated for 48 hours from days 5-7 of culture. (*Significantly different than control, $P<0.05$. +Significantly different than corresponding group without PD158780, $P<0.05$.) Each bar represents the mean ± SEM of triplicate culture wells. This graph is representative of 4 independent experiments. The slight inhibitory effect of PD158780 on thymidine incorporation in response to 40 ng/ml TNFα in this experiment was statistically insignificant in three other experiments.

Figure 2. The effects of EGF, TNFα and PD158780 on thymidine incorporation by MEC when measured on day 21 of culture. MEC were cultured in serum-free medium lacking EGF, TNFα and drug (control) until day 5; the medium was then changed and the MEC were treated as indicated for 16 days from days 5-21 of culture. (*Significantly different than control, $P<0.05$. +Significantly different than corresponding group without PD158780, $P<0.05$. #Significantly different than EGF-treated group, $P<0.05$.) Each bar represents the mean ± SEM of triplicate culture wells. This graph is representative of 2 independent experiments.

Figure 3. The effects of EGF, TNFα and PD158780 on viable cell number when measured on day 7 of culture. MEC were cultured in serum-free medium lacking EGF, TNFα and drug (control) until day 5; the medium was then changed and the MEC were treated as indicated for 48 hours from days 5-7 of culture. (*Significantly different than control, $P<0.05$. +Significantly different than corresponding group without PD158780, $P<0.05$.) Each bar represents the mean ± SEM of triplicate culture wells. This graph is representative of 3 independent experiments.

Figure 4. The effects of EGF, TNFα and PD158780 on viable cell number when measured on day 21 of culture. MEC were cultured in serum-free medium lacking EGF, TNFα and drug (control) until day 5; the medium was then changed and the MEC were treated as indicated for 16 days from days 5-21 of culture. (*Significantly different than control, $P<0.05$. +Significantly different than corresponding group without PD158780, $P<0.05$. #Significantly different than EGF-treated group, $P<0.05$.) Each bar represents the mean ± SEM of triplicate culture wells. This graph is representative of 3 independent experiments.

Figure 5. The effects of EGF, TNFα and PD158780 on the morphological appearance of MEC organoids in primary culture after 16 days of treatment. Control: a, squamous epithelial organoids; b, atypical organoid (hybrid of multilobular alveolar and squamous colonies); c, multilobular alveolar organoid. + PD158: d and e, multilobular alveolar organoids; note disrupted appearance. + EGF: f and g, multilobular alveolar organoids. + EGF + PD158: h and i, multilobular alveolar organoids; note disrupted appearance. + TNFα (40 ng/ml): j,

ductal-alveolar colony network. + TNFα (40 ng) + PD158: k, ductal-alveolar colony network; note the lack of effect of PD158 in the presence of TNFα. Magnification in all photographs is the same; bars = 100 μm.

Figure 6. The effects of EGF, TNFα and PD158780 on the morphological differentiation of MEC organoids in primary culture. The morphologic type of each colony was classified and quantitated on day 21 of culture. Four main colony types were quantitated: end bud-like, alveolar, squamous, and atypical hybrid, and the proportion of each is expressed as a percentage of total epithelial colonies. Bars represent the mean ± SEM of triplicate culture wells. (*Significantly different than control, $P<0.05$. +Significantly different than corresponding group without PD158780, $P<0.05$.) This graph is representative of 3 independent experiments.

Figure 7. The effects of EGF, TNFα and PD158780 on casein protein accumulation by MEC when measured on day 7 of culture. MEC were cultured in serum-free medium lacking EGF, TNFα and drug (control) until day 5; the medium was then changed and the MEC were treated as indicated for 48 hours from days 5-7. Equivalent amounts of protein (10 μg) from extracts of MEC plus RBM matrix were loaded into each lane and subjected to Western blot analysis for casein. Each lane represents a combination of triplicate wells for each group. This blot is representative of 3 independent experiments. A second Western blot in which loading was based on an equivalent number of cells in each lane was virtually identical (data not shown).

Figure 8. The effects of EGF, TNFα, and PD158780 on casein accumulation by MEC when measured on day 21 of culture. MEC were cultured in serum-free medium lacking EGF, TNFα and drug (control) until day 5; the medium was then changed and the MEC were treated as indicated for 16 days from days 5-21 of culture. A, Western blot analysis of casein protein accumulation in extracts of MEC plus RBM matrix. Loading was based on an equivalent number of cells (5×10^3) per lane, and each lane represents a combination of triplicate wells for each group. This blot is representative of 3 independent experiments. B, Determination of casein accumulation by ELISA. Casein levels in extracts of MEC plus RBM matrix were analyzed by ELISA, and results are expressed as nanograms per 10^5 cells. Bars represent the mean ± SEM of triplicate wells. (*Significantly different than control, $P<0.05$. +Significantly different than corresponding group without PD158780.) The absolute micrograms of casein per well (uncorrected for cell number) were as follows: 9.44 ± 2.08 (Control), 8.85 ± 1.04 (+PD158), 19.6 ± 8.37 (+EGF), 16.5 ± 1.26 (+EGF +PD158), 3.61 ± 1.43 (+TNFα 40 ng/ml), 2.45 ± 0.85 (+TNFα 40 ng +PD158), 12.3 ± 1.64 (+TNFα 2 ng/ml), 5.81 ± 0.79 (+TNFα 2 ng +PD158). This graph is representative of three experiments.

III. PUBLICATIONS

1. Varela, L.M., Ip, M.M. Tumor Necrosis Factor-α: A Multifunctional Regulator of Mammary Gland Development. Endocrinology *137*: 4915-4924, 1996.

2. Varela, L.M., Darcy, K.M., and Ip, M.M. The Epidermal Growth Factor Receptor is Not Required for TNFα Action in Normal Mammary Epithelial Cells. Endocrinology, *in press.*

IV. MEETING ABSTRACTS

1. M.M. Ip, L. Varela, S. Shoemaker, W. Shea, and K. Darcy. Regulatory role of tumor necrosis factor-α in mammary gland development. Presented at the International Association for Breast Cancer Research Meeting, Paris, France, July 1996.

2. L.M. Varela, K.M. Darcy and M.M. Ip. The epidermal growth factor receptor (EGFR) does not play a significant role in the pathway of TNFα action in normal mammary epithelial cells (MEC). Published in the *Proceedings of the Annual Meeting of the American Association of Cancer Research* **38**: A376, 1997.

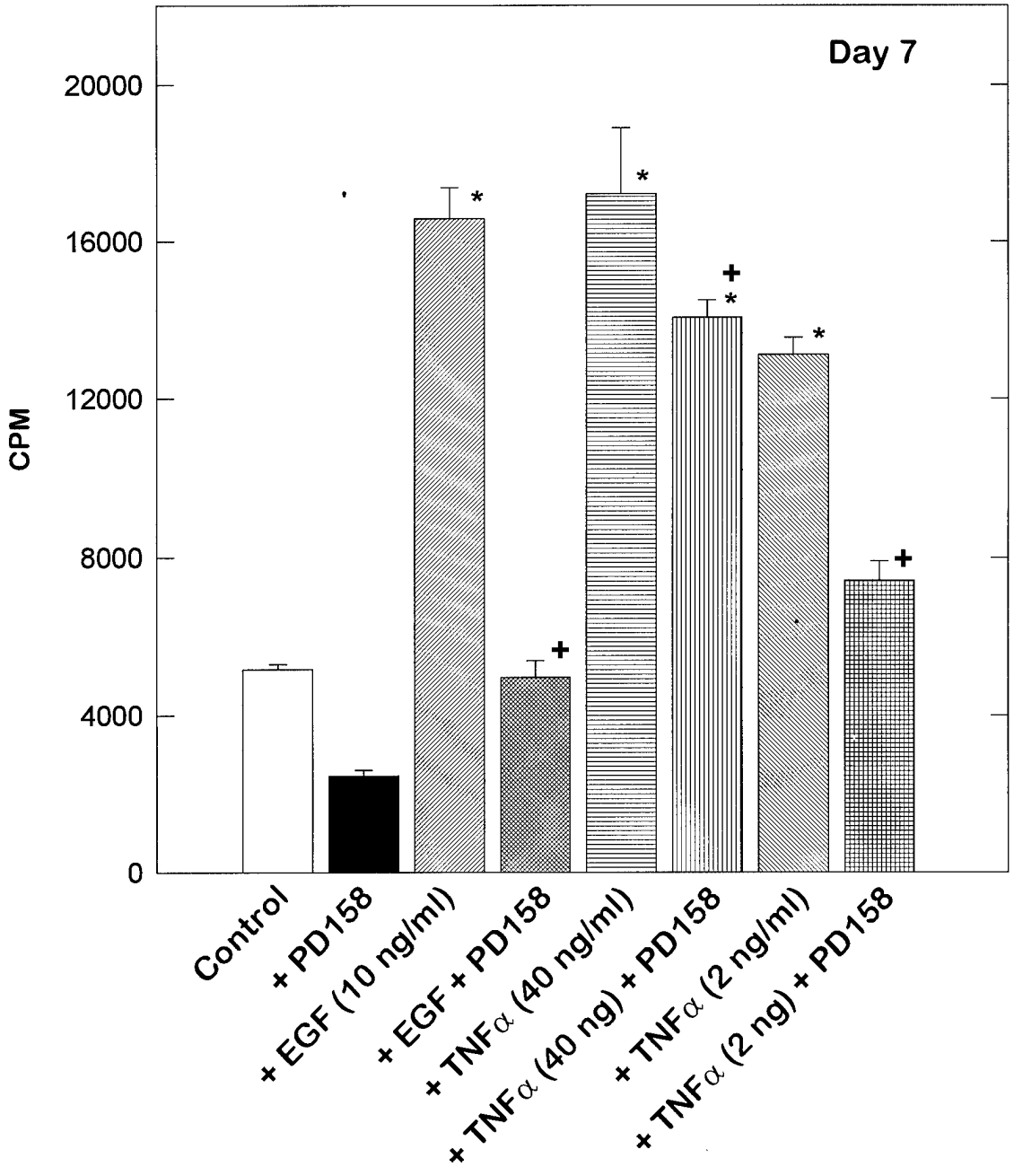
Day 7
CPM
20000
16000
12000
8000
4000
0
Control
+ PD158
+ EGF (10 ng/ml)
+ EGF + PD158
+ TNFα (40 ng/ml)
+ TNFα (40 ng) + PD158
+ TNFα (2 ng/ml)
+ TNFα (2 ng) + PD158

Figure 1.

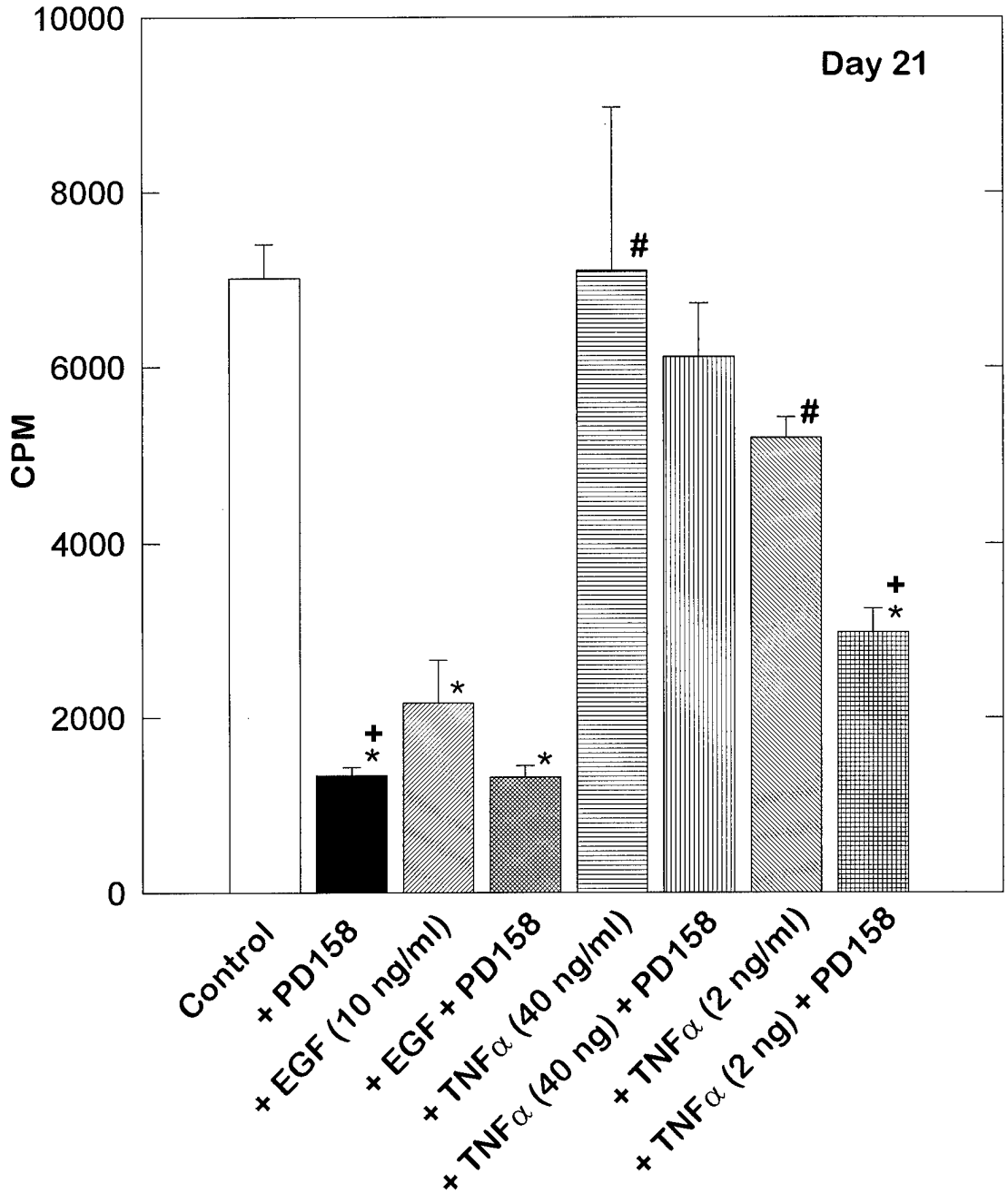
Day 21
CPM
10000
8000
6000
4000
2000
0
Control
+ PD158
+ EGF (10 ng/ml)
+ EGF + PD158
+ TNFα (40 ng/ml)
+ TNFα (40 ng) + PD158
+ TNFα (2 ng/ml)
+ TNFα (2 ng) + PD158
#
*
+

Figure 2.

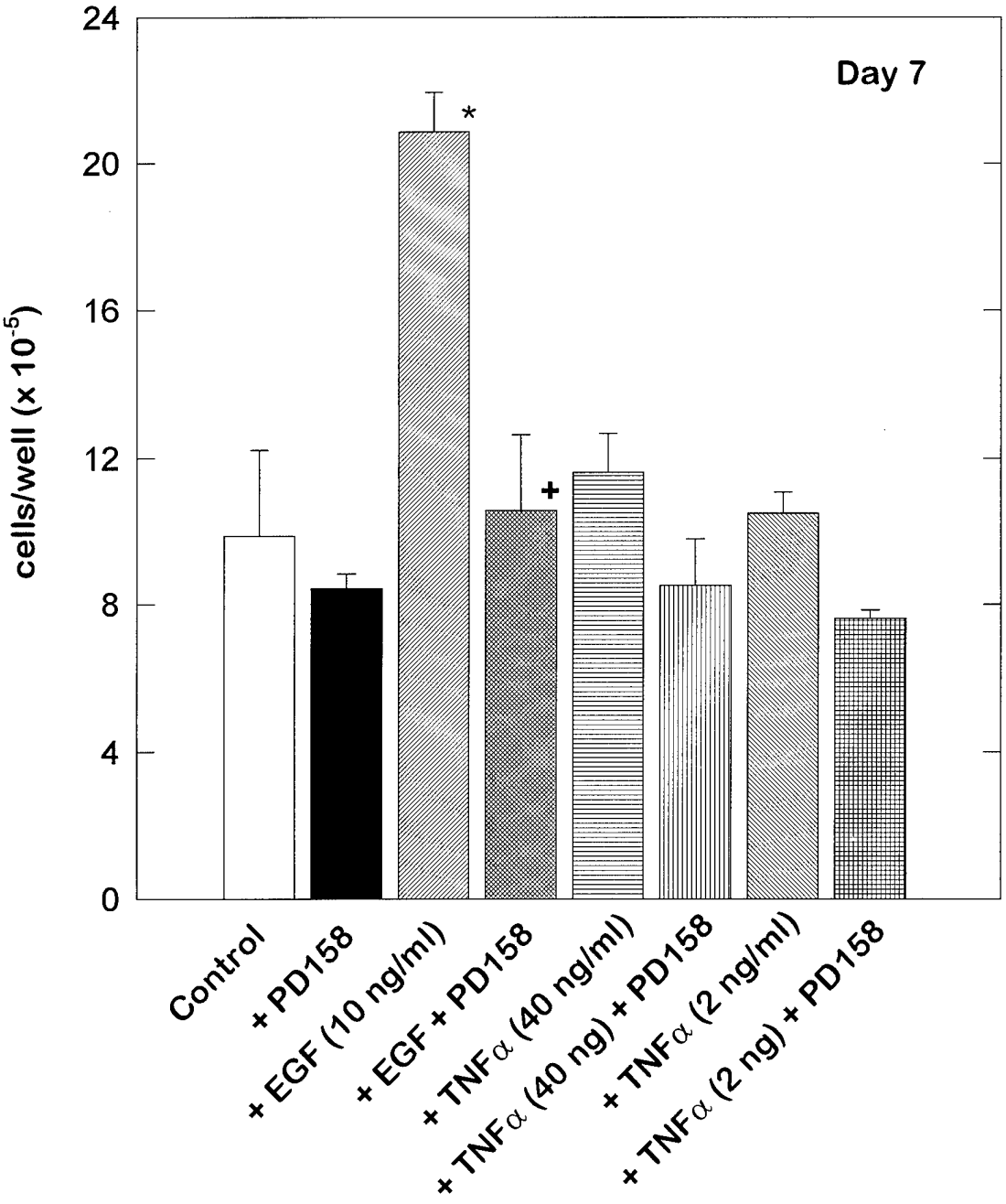
Day 7
cells/well (x 10^{-5})
24
20
16
12
8
4
0
*
+
Control
+ PD158
+ EGF (10 ng/ml)
+ EGF + PD158
+ TNFα (40 ng/ml)
+ TNFα (40 ng) + PD158
+ TNFα (2 ng/ml)
+ TNFα (2 ng) + PD158

Figure 3.

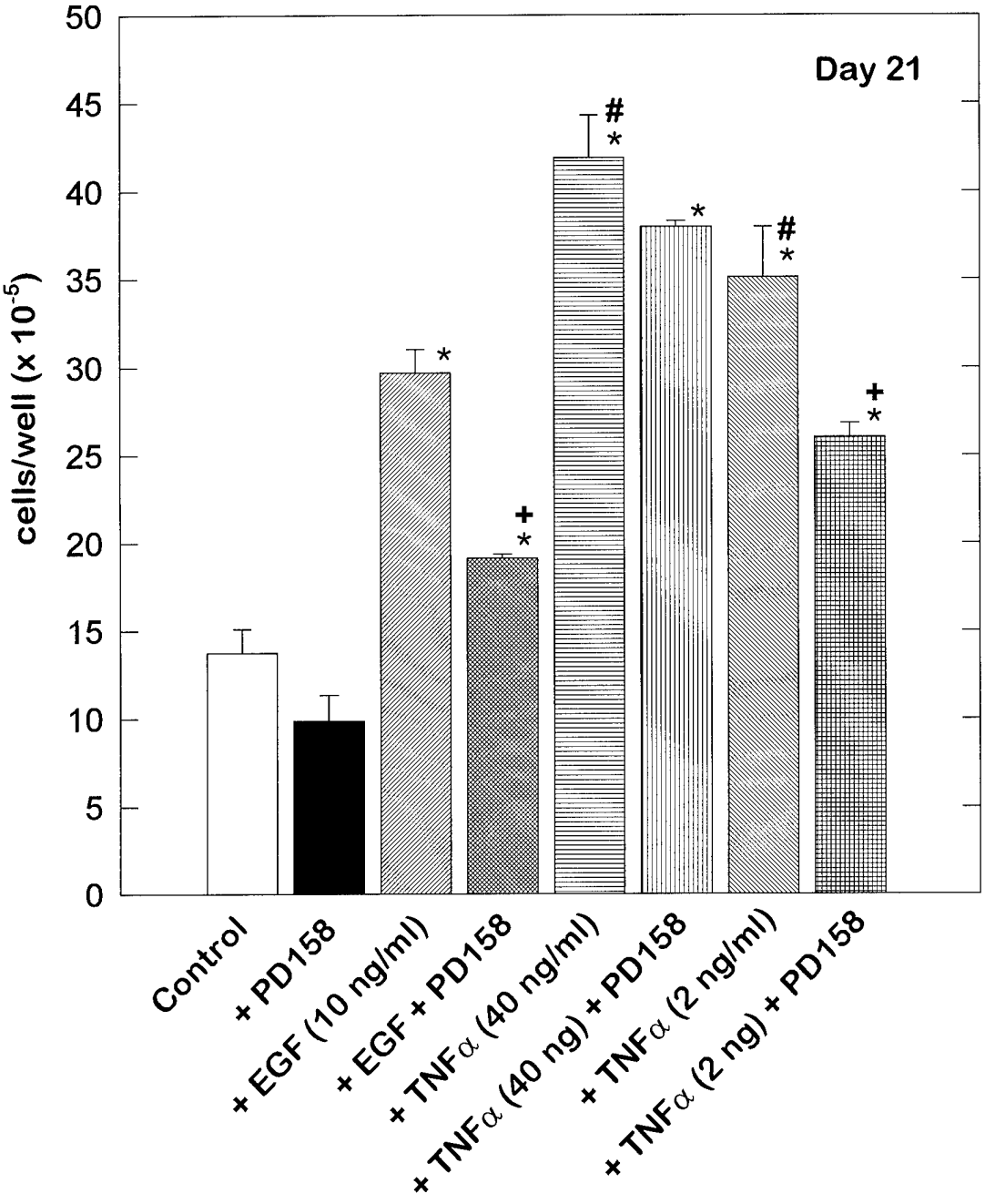
Day 21
cells/well (x 10^{-5})
50
45
40
35
30
25
20
15
10
5
0
Control
+ PD158
+ EGF (10 ng/ml)
+ EGF + PD158
+ TNFα (40 ng/ml)
+ TNFα (40 ng) + PD158
+ TNFα (2 ng/ml)
+ TNFα (2 ng) + PD158

Figure 4.

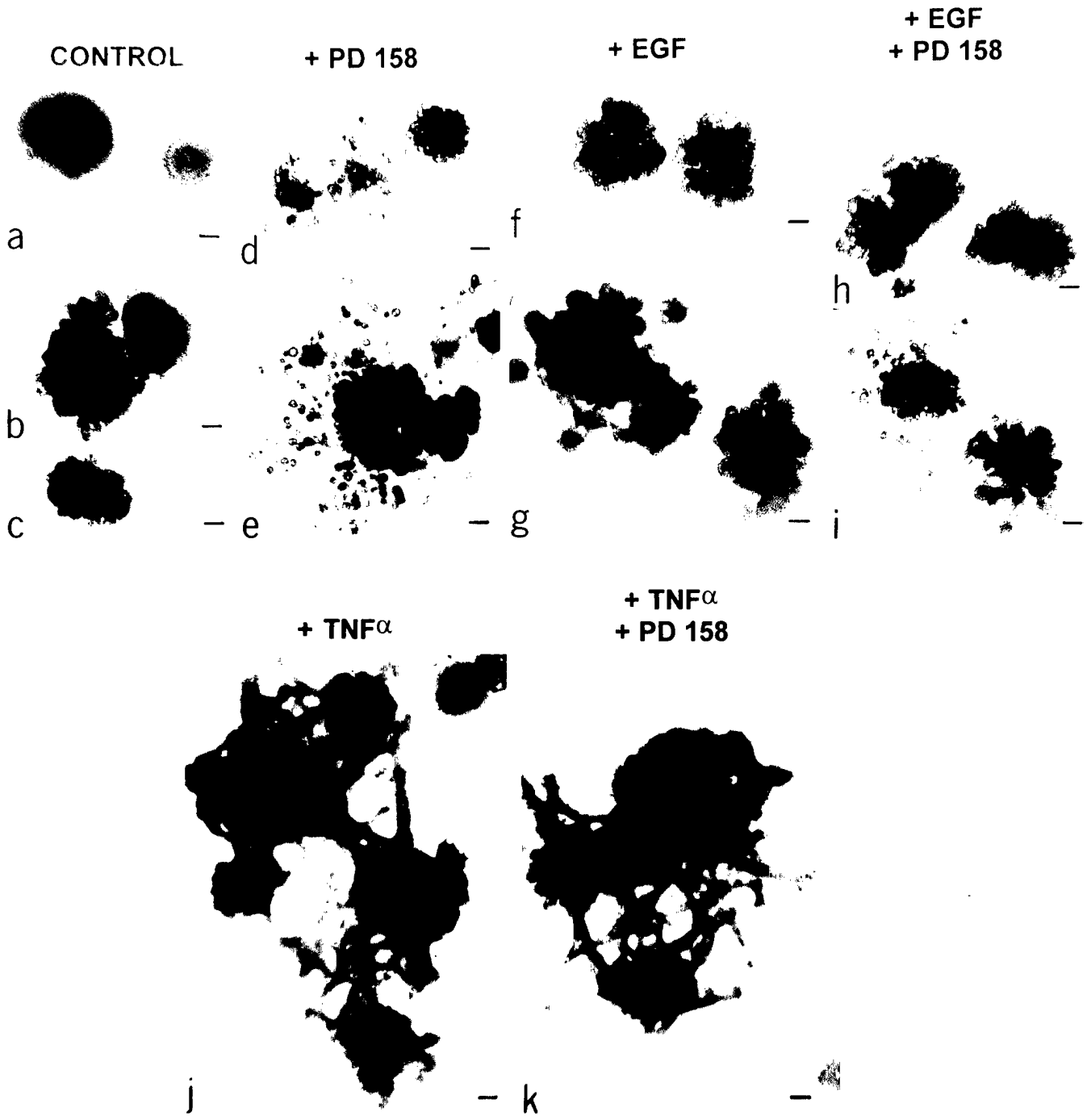

Figure 5.

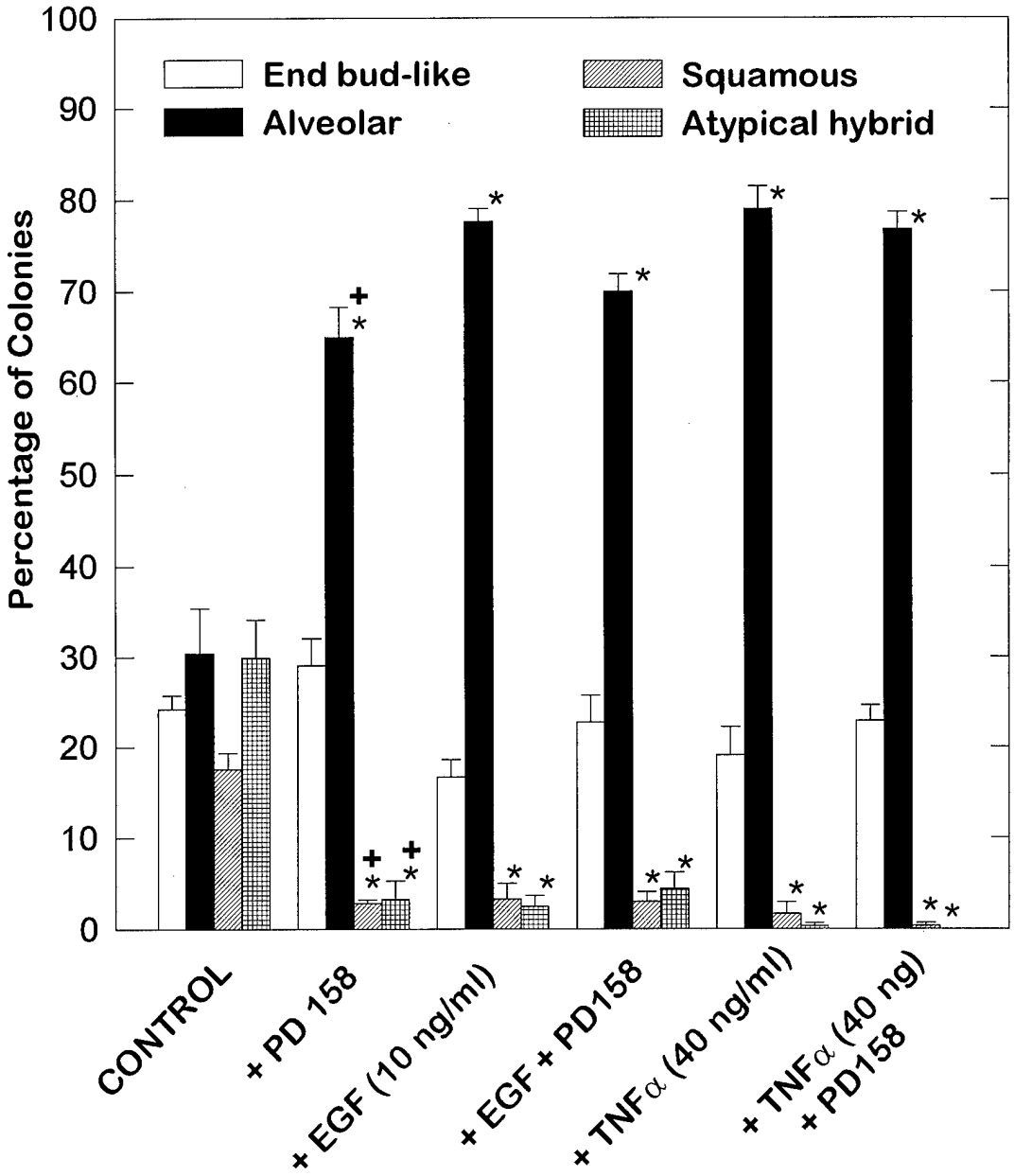
End bud-like
Alveolar
Squamous
Atypical hybrid
Percentage of Colonies
100
90
80
70
60
50
40
30
20
10
0
CONTROL
+ PD 158
+ EGF (10 ng/ml)
+ EGF + PD158
+ TNFα (40 ng/ml)
+ TNFα (40 ng)
+ PD158

Figure 6.

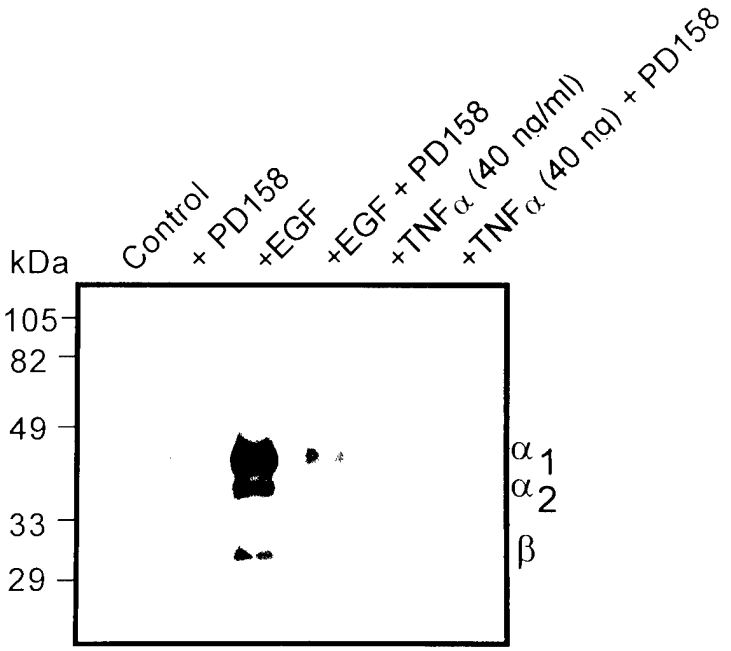
kDa
Control
+ PD158
+EGF
+EGF + PD158
+TNFα (40 ng/ml)
+TNFα (40 ng) + PD158
105
82
49
33
29
α1
α2
β

Figure 7.

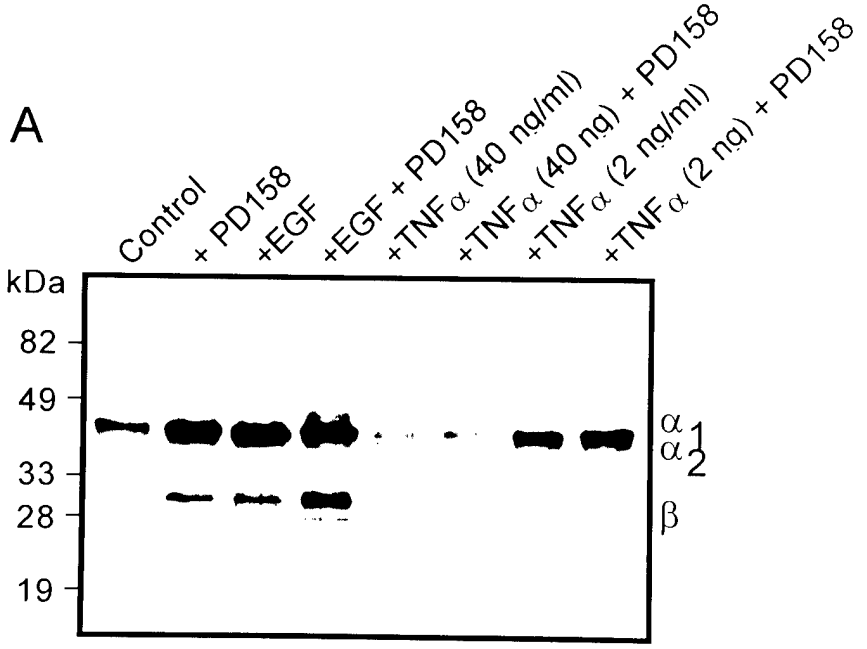
A
Control
+ PD158
+EGF
+EGF + PD158
+TNFα (40 ng/ml)
+TNFα (40 ng) + PD158
+TNFα (2 ng/ml)
+TNFα (2 ng) + PD158
kDa
82
49
33
28
19
α1
α2
β

Figure 8A.

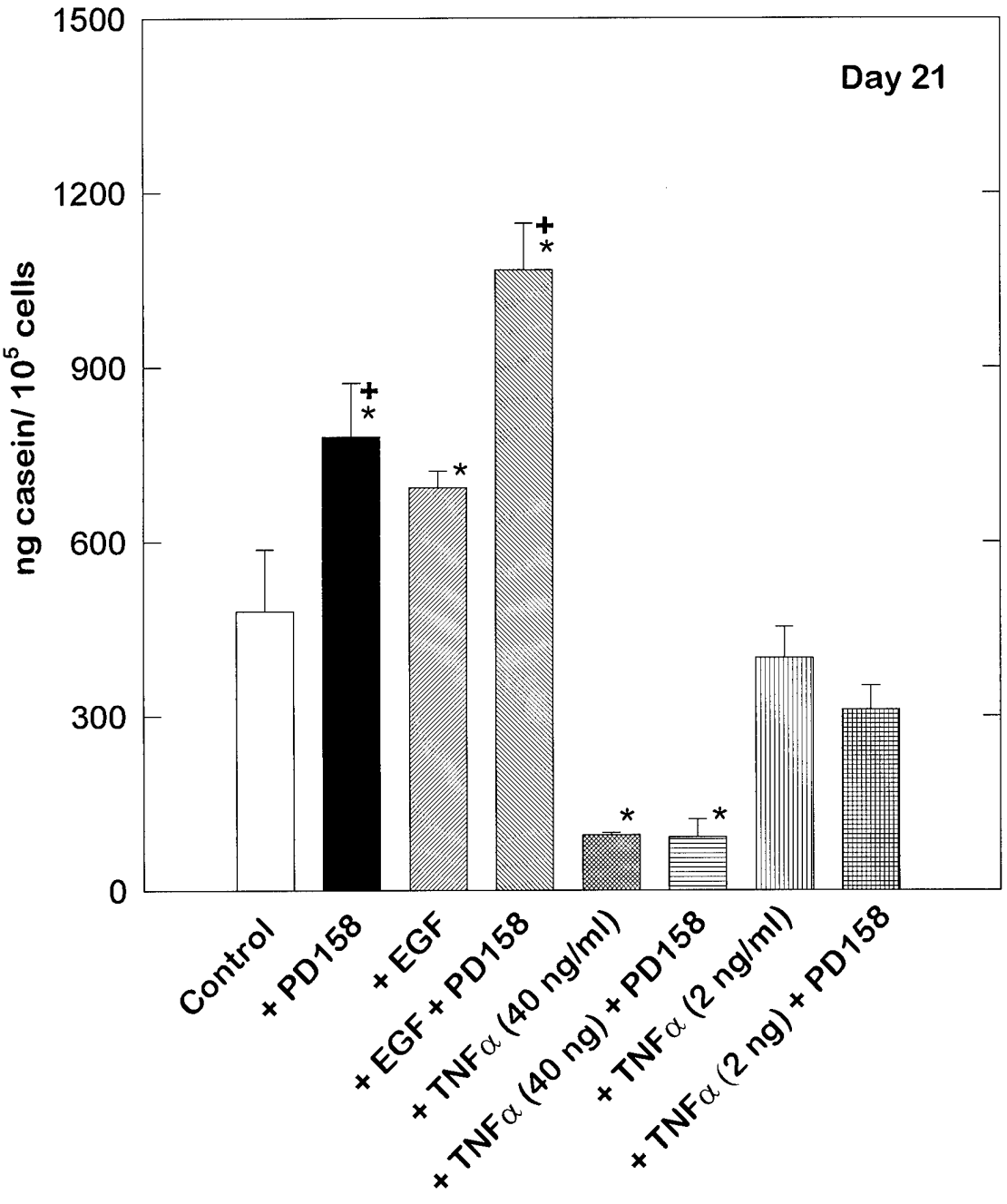
Day 21
ng casein/ 10^5 cells
1500
1200
900
600
300
0
Control
+ PD158
+ EGF
+ EGF + PD158
+ TNFα (40 ng/ml)
+ TNFα (40 ng) + PD158
+ TNFα (2 ng/ml)
+ TNFα (2 ng) + PD158

Figure 8B.